Mobilization of late-endosomal cholesterol is inhibited by Rab guanine nucleotide dissociation inhibitor

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Cholesterol entering cells in low-density lipoproteins (LDL) via receptor-mediated endocytosis is transported to organelles of the late endocytic pathway for degradation of the lipoprotein particles. The fate of the free cholesterol released remains poorly understood, however. Recent observations suggest that late-endosomal cholesterol sequestration is regulated by the dynamics of lysobisphosphatidic acid (LBPA)-rich membranes [1]. Genetic studies have pinpointed a protein, Niemann–Pick C-1 (NPC-1), that is required for the mobilization of late-endosomal/lysosomal cholesterol by an unknown mechanism [2]. Here, we report the removal of accumulated cholesterol by overexpression of the NPC-1 protein in NPC-1-deficient fibroblasts from patients with Niemann–Pick disease, and in normal fibroblasts upon release of a progesterone-induced block of cholesterol transport. We show that late-endosomal/lysosomal cholesterol mobilization is specifically inhibited by microinjection of Rab GDP-dissociation inhibitor (Rab-GDI). Moreover, clearance of the cholesterol deposits by NPC-1 in patients' fibroblasts is accompanied by the redistribution of LBPA and of a lysosomal hydrolase that utilizes the mannose-6-phosphate receptor. Our results reveal, for the first time, the involvement of a specific molecular component of the membrane-trafficking machinery in cholesterol transport and the coupling of late-endosomal cholesterol egress to the trafficking of other lipid and protein cargo.

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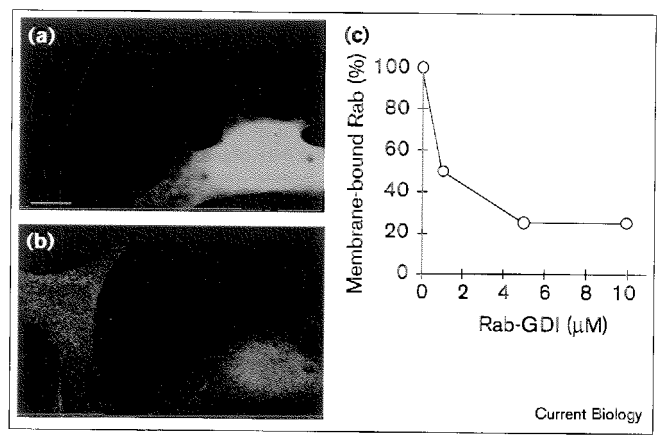
Results and discussion

Transport vehicles carrying LDL-derived cholesterol have not been characterized. Vesicle-mediated transfer would be consistent with the finding that sterol relocation from lysosomes can be blocked at 21°C [3]. However, several drugs that inhibit vesicular transport, for example cytoskeleton-disrupting agents, were found not to affect the movement of LDL-derived cholesterol (for review see [4]). To address whether the machinery functioning in the membrane trafficking of proteins is involved in cholesterol mobilization, we tested the effect of Rab-GDI, a specific inhibitory protein component of the membrane-trafficking apparatus, on lysosomal/late-endosomal cholesterol egress. Rab-GDI is required for the recycling of Rab GTPases that are involved in vesicle targeting and fusion along the exocytic and endocytic pathways (reviewed in [5]). We prepared recombinant Rab-GDI [6] and verified its activity in removing Rab proteins from membranes. Microinjection of human fibroblasts with Rab-GDI resulted in the redistribution of the late-endosomal marker Rab7 from its punctate perinuclear localization to a diffuse cytosolic localization (Figure 1a,b). Furthermore, Rab-GDI removed up to 75% of Rab proteins from isolated membranes (Figure 1c).

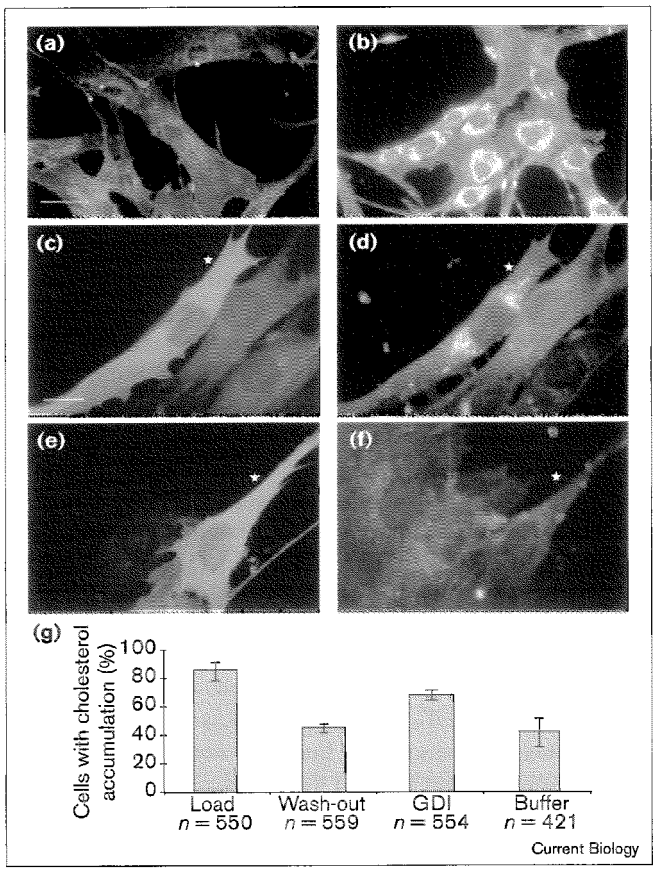
We then established conditions for monitoring cholesterol relocation in LDL-fed cells. Loading of fibroblasts with LDL in the presence of progesterone results in a cholesterol-trafficking block in the late endocytic organelles, which is rapidly reversed upon removal of progesterone [7].

Figure 1



Rab-GDI efficiently removes Rab proteins from membranes. (a,b) Human fibroblasts were microinjected with Rab-GDI and an IgG marker, incubated for 3 h before fixing, and stained with antibodies against (a) IgG and against (b) Rab7. The scale bar represents 20 μm. (c) The ability of Rab-GDI to remove Rab5 from membranes was determined as detailed in the Materials and methods.

Figure 2



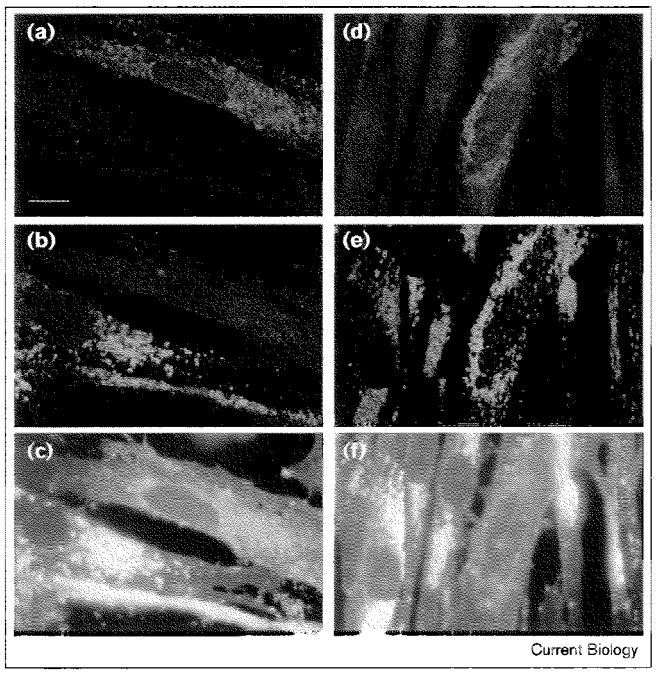
Removal of LDL-derived cholesterol is inhibited by microinjection of Rab-GDI in control fibroblasts. **(a–f)** Cholesterol-starved cells (a) were loaded with LDL in the presence of progesterone (b), and microinjected either with Rab GDI and IgG (c,d) or with IgG alone (e,f), and further incubated for 3–4 h without progesterone before fixing. The microinjected cells (asterisks) were visualized by fluorescein-conjugated anti-IgG antibodies (c,e) and free cholesterol by filipin staining (a,b,d,f). The scale bar represents 30 μm in (a) and 20 μm in (c). **(g)** For quantitation of cholesterol accumulation, non-injected cells (wash-out), cells injected with Rab-GDI and IgG (GDI) and cells injected with IgG (buffer) were scored by filipin staining after the wash-out period. As a control, cells were also stained immediately after LDL and progesterone loading (load). For quantitation of cholesterol accumulation, cells were viewed with a 40× objective and the characteristic appearance of multiple bright, perinuclear filipin-positive dots was scored as cholesterol accumulation. The number of cells analyzed from three to five individual experiments, and the standard deviations, are indicated.

We therefore treated fibroblasts with LDL in the presence of progesterone for 24 hours, fixed the cells either directly or after progesterone wash-out, and visualized the accumulation of unesterified cholesterol by light microscopy using the fluorescent antibiotic filipin (Figure 2a,b,d,f). After a wash-out period of 3–4 hours, the discharge of the intense filipin-positive deposits was clearly observed, whereas the faint filipin fluorescence characteristic of untreated cells at the perinuclear area and on the plasma membrane remained (Figure 2d,f, non-injected cells). When the cells were microinjected with Rab-GDI at the initiation of the wash-out, however, several deposits brightly stained with filipin remained after the wash-out period, indicating a block in cholesterol mobilization (Figure 2c,d). This inhibition was not observed when only the co-injection marker IgG was injected in the same buffer (Figure 2e,f). Quantitation revealed that Rab-GDI inhibited cholesterol relocation by 50% (Figure 2g). Although prolonging the wash-out reduced the number of cells with cholesterol accumulation, the wash-out time was limited by the notion that Rab-GDI loses its effect in the cellular environment with time (data not shown). The degree of transport inhibition by Rab-GDI was in the range previously observed on introduction of GDI into living cells [8–11].

Massive lysosomal deposits of unesterified cholesterol can be found in the fibroblasts of patients suffering from the genetic disease Niemann–Pick C (NPC). These accumulations can be removed by introducing the normal NPC-1 gene product into the cells [2]. We next determined whether the cholesterol transport that resumes in NPC cells after the release of the genetic block is also sensitive to Rab-GDI. NPC-1-deficient fibroblasts were co-transfected with cDNAs for NPC-1 and green fluorescent protein (GFP), and progesterone was added 24 hours after transfection in order to prevent asynchronous cholesterol mobilization from cells expressing slowly increasing levels of NPC-1. Three days after transfection, the transfected cells (identified by GFP fluorescence) were microinjected with Rab-GDI, and cholesterol mobilization was analyzed as above. We found that introduction of Rab-GDI into the transfected cells significantly inhibited complementation of the defect by NPC-1. The cholesterol accumulations remained in 50% of the transfected cells injected with Rab-GDI but in only 20–25% of transfected and control-injected or non-injected cells; the efficiency of transport inhibition by Rab-GDI was comparable to that in normal cells loaded with LDL and progesterone (data not shown). Together, these results suggest that cholesterol is mobilized from the late-endosomal membranes to carriers that rely on Rab proteins for their delivery.

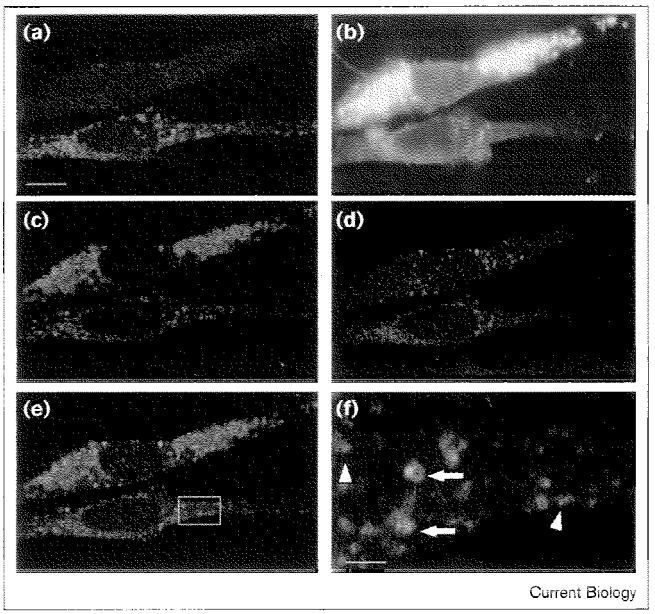
In our experiments, the outcome of removing Rab proteins from their functional cycle by excess Rab-GDI was stagnation of the sterol cargo to the donor membranes. Considering our current understanding of Rab proteins as regulators of the docking and fusion steps of vesicular transport, the anticipated effect would rather have been unsuccessful fusion of transport intermediates to the sites of cargo delivery. The observed retention of the late-endosomal cholesterol might reflect an indirect effect of exhausting the pool of specific Rab proteins required for the transfer process. Moreover, the role of Rab proteins might be more complex than simply involvement in vesicle targeting and fusion. A complex containing Rab5

Figure 3



Redistribution of LBPA upon cholesterol removal in NPC fibroblasts. Confocal micrographs of NPC-1-deficient cells transfected with NPC-1 cDNA and stained as follows. Staining with antibodies against **(a)** NPC-1 and **(b)** LBPA; **(c)** filipin staining of the cells in (a,b). Staining with antibodies against **(d)** NPC-1 and **(e)** Lamp-1; **(f)** filipin staining of cells in (d,e). The scale bar in (a) represents 20 μm.

Figure 4



The redistribution of the lysosomal hydrolase AGA in NPC fibroblasts upon cholesterol removal. **(a–f)** Confocal micrographs of NPC-1-deficient cells transfected with Myc-tagged NPC-1 cDNA and stained with antibodies against (a) the Myc tag, (c,e,f) against AGA (green) and (d–f) against EEA1 (red). (b) Cholesterol was visualized by filipin staining. (e) Overlay of (c) and (d). (f) The area indicated in (e) at higher magnification. Yellow indicates co-localization (arrows); compare with red EEA1 staining and green AGA staining (arrowheads). The scale bar represents 20 μm in (a) and 5 μm in (f).

and GDI was recently shown to be required in the vesicle formation stage [12]. Furthermore, GDI inhibits not only vesicular transport but also direct membrane fusion between late endosomes and lysosomes [13].

Immunoelectron microscopy has revealed that some constituents of the late endosomes are confined to the encircling membrane (for example, Lamp-1), whereas others (for example, the mannose-6-phosphate receptor (MPR) and some lysosomal hydrolases that utilize this receptor) localize within the internal membranes [14]. LBPA is also found in the internal membranes [15]. As these membranes are also heavily loaded with cholesterol [16], unpublished observations), we determined whether any other constituents of the late-endosomal membranes would be mobilized along with cholesterol by analyzing the distribution of late-endosomal/lysosomal markers in NPC fibroblasts complemented with the NPC-1 protein. We found that in cells in which the cholesterol accumulation was efficiently removed, the punctate late-endosomal staining typical of LBPA in control and NPC fibroblasts [1] changed dramatically to a diffuse staining pattern (Figure 3a–c). In contrast, the distribution of Lamp-1 was not altered and it continued to co-localize with the NPC-1 protein, which is localized in the late-endosomes/lysosomes handling the sterol load [3] (Figure 3d–f). We also studied the distribution of the soluble lysosomal hydrolase aspartylglucosaminidase (AGA), which uses the mannose 6-phosphate recognition tag [17], in the complemented NPC cells. Interestingly, whereas AGA overlaps extensively with Lamp-1 in both normal fibroblasts and uncorrected NPC cells (data not shown), it became partially redistributed in the corrected NPC cells, as reflected by a change from the characteristic punctate, often perinuclear, ring-like staining to a more scattered pattern (Figure 4a–c). Several of the AGA-positive signals distributed towards the cell periphery co-localized with the early endosomal autoantigen EEA1 (Figure 4d–f), indicating that AGA was partially targeted to early endosomes upon cholesterol mobilization.

No relocation of LBPA and AGA was observed when NPC-1 was overexpressed in normal fibroblasts (data not shown). This suggests that the redistribution of the late endosomal components observed in NPC fibroblasts could be due to missorting or to an alteration in the balance between forward transport and retrieval of cargo in the cholesterol-loaded organelles. The differential sensitivity of the late-endosomal components to redistribution might reflect the hierarchical read-out of their sorting information: whereas Lamp-1 contains a 'dominant' dileucine

motif for lysosomal targeting [18], LBPA might rely on weaker ionic interactions for correct localization.

Our results reveal that late-endosomal cholesterol egress is coupled to the membrane trafficking of other lipid and protein cargo from that compartment. The question then arises as to the destination of the cholesterol-containing carriers. The bulk of lysosomal cholesterol is thought to be mobilized to the plasma membrane [19,20]. There is also evidence for the transit of lysosomally derived cholesterol via the Golgi complex [16]. An alternative route independent of the plasma membrane is, however, suggested to transport cholesterol from the lysosomes to the endoplasmic reticulum [20]. The identification of Rab proteins as regulators of the cholesterol-trafficking circuits now offers the possibility of manipulating these compartment-specific molecular switches to dissect the routes taken by endocytosed cholesterol.

Materials and methods

The 93.41 NPC fibroblasts were provided by Peter Pentchev (NIH) and F92-99 control fibroblasts by Harriet von Koskull (Helsinki University Central Hospital). For cholesterol loading, control fibroblasts seeded on coverslips were cultured in medium supplemented with 5% lipoprotein-deficient calf serum for 24–72 h. The cells were then transferred into medium supplemented with 50 μg/ml human LDL and 10 μg/ml progesterone for an additional 22–24 h before microinjection. Recombinant His_6-tagged Rab-GDI (8 mg/ml) was prepared and its activity measured as described previously [6], using the postnuclear supernatant fraction of CHO cells. Rab-GDI was injected into the cytoplasm at 6.4 mg/ml and mouse monoclonal anti-human IgG at 0.8 mg/ml. NPC fibroblasts were seeded on coverslips 1 day before transfection at 50–80% confluency, and transfected using FUGENE6 transfection reagent (Boehringer–Mannheim). For immunofluorescence microscopy, cells fixed with 4% paraformaldehyde were incubated in 10% fetal bovine serum (FBS) supplemented with 0.05% filipin, followed by primary antibodies diluted in 5% FBS/0.05% filipin, and secondary antibodies diluted in 5% FBS. The coverslips were viewed with a Zeiss Axiophot photomicroscope or with a Leica TCS NT confocal microscope.

Supplementary material

Supplementary material containing more detailed information on the plasmids and antibodies used, and additional methodological details is available at http://current biology.com/supmat/supmatin.htm.

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